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Review

Vaccines and animal models for arboviral encephalitides

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Abstract

Arthropod-borne viruses ("arboviruses") cause significant human illness ranging from mild, asymptomatic infection to fatal encephalitis or hemorrhagic fever. The most significant arboviruses causing human illness belong to genera in three viral families, Togaviridae, Flaviviridae, and Bunyaviridae. These viruses represent a significant public health threat to many parts of the world, and, as evidenced by the recent introduction of the West Nile virus (WNV) to the Western Hemisphere, they can no longer be considered specific to any one country or region of the world. Like most viral diseases, there are no specific therapies for the arboviral encephalitides; therefore, effective vaccines remain the front line of defense for these diseases. With this in mind, the development of new, more effective vaccines and the appropriate animal models in which to test them become paramount. In fact, for many important arboviruses (e.g. California serogroup and St. Louis encephalitis viruses), there are currently no approved vaccines available for human use. For others, such as the alphaviruses, human vaccines are available only as Investigational New Drugs, and thus are not in widespread use. On the other hand, safe and effective vaccines against tick-borne encephalitis virus (TBEV) and Japanese encephalitis virus (JEV) have been in use for decades. New challenges in vaccine development have been met with new technologies in vaccine research. Many of the newer vaccines are now being developed by recombinant DNA technology. For example, chimeric virus vaccines have been developed using infectious clone technology for many of the arboviruses including, WNV, JEV, and TBEV. Other successful approaches have involved the use of naked DNA encoding and subsequently expressing the desired protective epitopes. Naked DNA vaccines have been used for TBEV and JEV and are currently under development for use against WNV. The development of less expensive, more authentic animal models to evaluate new vaccines against arboviral diseases will become increasingly important as these new approaches in vaccine research are realized. This article reviews the current status of vaccines, both approved for use and those in developmental stages, against the major arboviral encephalitides causing human disease. In addition, research on animal models, both past and present, for these diseases are discussed.

Keywords: Vaccines; Arboviral encephalitides; Infectious diseases

1. Introduction

Diseases caused by arboviruses are among the most important emerging infectious disease, public health problems facing the world today (Gubler, 2001). These viruses belong to taxonomically diverse groups representing at least 8 viral families and 14 genera (Gubler and Roehrig, 1998). There are currently 534 viruses registered in the International Catalogue of Arboviruses, of which 134 are known to cause disease in humans and approximately 40 that infect livestock (Karabatsos, 1985). The most significant arboviruses causing human illness belong to the viral families, Togaviridae, Flaviviridae, and Bunyaviridae. These viruses cause a variety of symptoms in infected humans. The clinical spectrum of disease can include subclinical infection, systemic febrile illness, arthralgia, febrile myalgia, encephalomyelitis, or hemorrhagic fever. The same virus may also produce different syndromes in different individuals depending on host factors such as age and/or immunological status.

The arboviruses that cause meningoencephalitis and/or encephalomyelitis are among the most serious and often produce a fatal outcome or permanent neurological sequelae. These viruses have a marked neurotropism, which leads to the characteristic pathological disease state. The arboviruses most frequently causing encephalitis in humans are listed in Table 1. Infections with these viruses can produce symptoms that include a sudden fever, vomiting, stiff neck, dizziness, drowsiness, disorientation, confusion, and progression

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14. ABSTRACT

Arthropod-borne viruses ("arboviruses") cause significant human illness ranging from mild, asymptomatic infection to fatal encephalitis or hemorrhagic fever. The most significant arboviruses causing human illness belong to genera in three viral families, Togaviridae, Flaviviridae, and Bunyaviridae. These viruses represent a significant public health threat to many parts of the world, and, as evidenced by the recent introduction of the West Nile virus (WNV) to the Western Hemisphere, they can no longer be considered specific to any one country or region of the world. Like most viral diseases, there are no specific therapies for the arboviral encephalitides; therefore, effective vaccines remain the front line of defense for these diseases. With this in mind, the development of new, more effective vaccines and the appropriate animal models in which to test them become paramount. In fact, for many important arboviruses (e.g. California serogroup and St. Louis encephalitis viruses), there are currently no approved vaccines available for human use. For others, such as the alphaviruses, human vaccines are available only as Investigational New Drugs, and thus are not in widespread use. On the other hand, safe and effective vaccines against tick-borne encephalitis virus (TBEV) and Japanese encephalitis virus (JEV) have been in use for decades. New challenges in vaccine development have been met with new technologies in vaccine research. Many of the newer vaccines are now being developed by recombinant DNA technology. For example, chimeric virus vaccines have been developed using infectious clone technology for many of the arboviruses including, WNV, JEV, and TBEV. Other successful approaches have involved the use of naked DNA encoding and subsequently expressing the desired protective epitopes. Naked DNA vaccines have been used for TBEV and JEV and are currently under development for use against WNV. The development of less expensive, more authentic animal models to evaluate new vaccines against arboviral diseases will become increasingly important as these new approaches in vaccine research are realized. This article reviews the current status of vaccines, both approved for use and those in developmental stages, against the major arboviral encephalitides causing human disease. In addition, research on animal models, both past and present, for these diseases are discussed.

15. SUBJECT TERMS

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Table 1
Major arboviruses that cause encephalitis

Family/virus	Principle vertebrate host	Arthropod vector	Geographic distribution	Vaccine[a]
Togaviridae				
Eastern equine encephalitis	Birds	*Culiseta*, *Culex* mosquitoes and other species	North and South America	IND
Venezuelan equine encephalitis	Rodents	*Aedes*, *Culex* mosquitoes and other species	Central and South America, southern Florida	IND
Western equine encephalitis	Birds, jackrabbits	*Culex* mosquitoes	North and South America	IND
Flaviviridae				
Japanese encephalitis	Birds, swine	*Culex* mosquitoes	Asia, India, far-eastern former Soviet Union	Licensed
Louping ill	Birds		Britain	
Murray Valley encephalitis	Birds	*Culex* mosquitoes	Australia, New Guinea	None
Powassan	Rodents, rabbits, opossums	*Ixodes*, *Dermacentor*, *Haemaphysalis* ticks	Russia, North America	None
Rocio	Birds	*Culex* mosquitoes	Brazil	None
St. Louis encephalitis	Birds	*Culex* mosquitoes	North and South America	None
Tick-borne encephalitis	Rodents	*Ixodes*, *Dermacentor*, *Haemaphysalis* ticks	Europe, Russia, former Soviet Union	
West Nile	Birds	*Culex* mosquitoes and other species	Eurasia, Africa, North America	None
Bunyaviridae				
California encephalitis	Rodents, jackrabbits, cottontail rabbits	*Ochlerotatus* and *Aedes* mosquitoes	Western North America	None
Jamestown Canyon	Deer	*Culiseta* and *Ochlerotatus* mosquitoes	North America	None
La Crosse encephalitis	Chipmunks, squirrels	*Ochlerotatus* mosquitoes	North America	None
Snowshoe hare	Rabbits	*Ochlerotatus* and *Culiseta* mosquitoes	North America	None

[a] Vaccine status of human vaccines in the US only; IND: Investigational New Drug status.

to coma and death. In some cases, recovery may leave neurological sequelae such as neuropsychiatric symptoms in adults or mental retardation in children. In addition, paralysis of the extremities can result from infection with certain arboviruses (e.g. TBEV) (Haglund and Gunther, 2003).

Is there a need for vaccines against arboviruses? There are many potential reasons why a biopharmaceutical company may not want to develop human vaccines against arboviral diseases. Most of these reasons relate to the low incidence of disease, the high cost-to-benefit ratio, and the high cost of vaccine development including the required preclinical and clinical trials. While the economic reasons are valid concerns and arboviral infections are relatively rare, the clinical disease (e.g. encephalitis) is severe and potentially fatal. Additionally, there are certain populations who are at higher risk, such as the elderly or infirm; residents in areas with high viral transmission; or people that have the potential for occupational exposure (i.e. laboratory workers), who would certainly benefit from vaccination. In fact, the U.S. Army has developed a number of arbovirus vaccines intended primarily for protecting military troops from biological warfare and naturally occurring disease. To date, the primary use of many of these vaccines has been to protect laboratory personnel working in research laboratories. There are a number of other factors that justify the investment in development of vaccines for arboviral infections. Unlike many bacterial infections, there are no or very few effective drugs approved for treating arboviral encephalitis (Bray and Huggins, 1998). There is increasing concern and fear regarding the use of chemical pesticides for vector control. In addition, considerable potential exists for economic losses related to arboviral disease in animals. As an example, in the case of West Nile virus (WNV), there is concern for loss of endangered species (e.g. exotic bird species in zoos) and there has been a significant impact on the horse population from WNV infection.

2. Bunyaviridae

The bunyaviruses are lipid-enveloped single-stranded RNA viruses that form spherical particles 80–120 nm in diameter. These viruses share a common genetic organization of three negative-stranded RNA segments termed S, M, and L (small, medium, and large). With the exception of the hantaviruses, which are transmitted by rodents, most of the bunyaviruses are arthropod-borne. More than 150 viruses and 16 serogroups are classified in the genus *Bunyavirus*.

The most important of the arthropod-borne bunyaviruses that produce encephalitis in humans belong to the California serogroup and include California encephalitis virus (CEV), La Crosse virus (LACV), Jamestown Canyon virus (JCV), and snowshoe hare virus (Nichol, 2001; Bray and Huggins, 1998). The California serogroup virus infections are the most commonly reported cause of arboviral encephalitis in the United States. Symptoms range from inapparent or mild febrile disease to encephalitis and death (Thompson et al., 1965; McJunkin et al., 1998). After a 3–7-day incubation period, sudden onset of fever, followed by stiff neck, lethargy, headache, nausea, and vomiting may be observed in infected individuals. Seizures have been seen in approximately half of the infected patients, and about 65% of the adult patients exhibit signs of meningitis. Seizures are the most important sequelae in children and have been observed in approximately 10–15% of children 1–8 years after infection (Chun et al., 1968; Grabow et al., 1969; Chun, 1983; McJunkin et al., 1998).

La Crosse virus was first isolated in La Crosse, Wisconsin in 1960 from the brain of a child who died from encephalitis (Thompson et al., 1965). It is related to CEV, but distinct enough to be considered another member of California serogroup. LACV causes encephalitis primarily in children. More than 80% of patients with encephalitis recover without any residual effects of central nervous system (CNS) infection. Epilepsy is the most important sequela and only occurs in about 10% of the patients (Gonzalez-Scarano et al., 1991). *Aedes triseriatus* mosquitoes are the primary vector for LACV and are found throughout the northern midwest and northeastern states in the US. These mosquitoes maintain the virus by transovarial transmission in which the virus persists in mosquito eggs during the winter. During the summer, LACV amplifies horizontally in a cycle among small mammals such as squirrels, chipmunks, foxes, and woodchucks (Thompson, 1983; Yuill, 1983; Balkhy and Schreiber, 2000). Snowshoe hare virus is an antigenic variant of LACV and is mostly seen in *Aedes* mosquitoes throughout Canada. It is a rare cause of human encephalitis mainly in Nova Scotia, Quebec, and Ontario (Gonzalez-Scarano et al., 1991).

Jamestown Canyon virus was first isolated from a pool of mosquitoes in Colorado in 1961 (Grimstad, 1988). In humans, JCV causes an encephalitis that resembles that seen in LACV encephalitis. In contrast to LACV, JCV primarily causes encephalitis in adults. *Culiseta inornata* and several species of *Aedes* mosquitoes, which are found across North America, are the principal vectors for JCV. Studies have demonstrated that the virus is vertically transmitted in several *Aedes* species mosquitoes (Hardy et al., 1993). Humans, white-tailed deer, mule deer, moose, horses, and rabbits are the major vertebrate hosts.

Although California serogroup viruses infect several animal species, including rabbits and rats, the laboratory mouse is the preferred animal model with which to study the pathogenesis of these viruses. Subcutaneous infection of newborn mice with these viruses very closely mimics the natural human infection and thus provides a good laboratory model to study viral pathogenesis and disease manifestation (Johnson, 1983; Janssen et al., 1984; Gonzalez-Scarano et al., 1991). However, the resulting pathogenesis of CEV, LACV, and JCV infections varies in the preferred animal model depending on the age of the mice and the particular strain of virus. LACV virus spreads to the CNS and produces encephalitis only in immature mice. After subcutaneous inoculation, the virus replicates primarily in striated muscle tissue, and then travels through the lymphatic circulation where the virus spreads to plasma and subsequently to the CNS. Death occurs in 72–96 h postinfection and is usually preceded by encephalitis. The virus replicates in neurons and glial cells and causes neuronal necrosis, cerebral edema, perivascular cuffing, glial nodules, and mild leptomeningitis which are typical signs of viral encephalitis (Johnson, 1983). As mice age, they become decreasingly susceptible to peripheral infection and studies have shown that disease in adult mice results in fatality only after intracerebral inoculation of LACV (Janssen et al., 1984). After intracerebral inoculation of adult mice, decreased activity and, occasionally, seizures are observed and death occurs by 5–6 days postinfection (Johnson, 1983). Studies have also demonstrated transplacental transmission of LACV in domestic rabbits and Mongolian gerbils. LACV infection of both pregnant gerbils and rabbits resulted in in utero and neonatal mortality (Osorio et al., 1996).

The lack of good animal models severely limits the development of vaccines against California serogroup viruses; currently, there are no approved vaccines for these viruses. Because LACV infects and causes encephalitis only in newborn mice and mice become resistant to infection as adults, there is a need to develop new animal models to test potential vaccines and antiviral drugs. Recently, there have been attempts to develop a DNA vaccine against LACV by using interferon type I (IFNAR-1) knockout mice (Schuh et al., 1999; Pavlovic et al., 2000). Previous studies showed that IFNAR-1 knockout mice are highly susceptible to infections with LACV and develop encephalitis regardless of their age (Muller et al., 1994; Hefti et al., 1999). In a recent study, vaccination with a plasmid containing the viral surface glycoproteins G1 and G2 protected IFNAR-knockout mice from challenge with LACV (Schuh et al., 1999).

3. Flaviviridae

Viruses in the family Flaviviridae are spherical, lipid-enveloped, and contain a positive-sense, single-stranded RNA genome. All members of the genus Flavivirus are antigenically related and distinct serocomplexes are defined on the basis of cross-neutralization tests (de Madrid and Porterfield, 1974). In addition, flaviviruses can be divided into three biological subsets based on their mode of transmission: tick-borne, mosquito-borne, or those having no known vector (Kuno et al., 1998).

3.1. Tick-borne encephalitis virus

Tick-borne encephalitis (TBE) was first described by an Austrian physician in 1931. In 1937 a virus was isolated from the brain of an encephalitis patient in the southern far east region of Russia and was named Russian spring-summer encephalitis (RSSE) due to its seasonal periodicity; it was later shown to be transmitted to humans by ticks (Gresikova and Calisher, 1988). The disease was first recognized in eastern Europe during an epidemic in 1948, and a virus isolated from a patient was shown to be similar to the far-eastern virus (i.e. RSSE) and subsequently named Central European encephalitis (CEE) virus. RSSE and CEE viruses are antigenically closely related and are now considered to be subtypes of the same virus (i.e. TBEV). However, there are a number of distinctions between the RSSE and CEE viruses. They are transmitted by two different tick vectors, RSSE by *Ixodes persulcatus* and CEE by *Ixodes ricinus*. In addition to their different geographical distribution, RSSE virus generally causes a more severe disease than does CEE virus. Additionally, RSSE and CEE viruses can be distinguished by cross-neutralization (Calisher et al., 1989) and by other serological assays (Calisher, 1988). Some investigators have proposed the existence of a third subtype (Siberian subtype) based on phylogenetic analysis of the envelope (E) protein to encompass the central Siberian strains, Aina and Vasilchenko (Ecker et al., 1999). TBEV is a member of a group of antigenically related viruses isolated from many areas across Eurasia and Canada. Historically, this group has been referred to as TBEV serocomplex or antigenic complex (Porterfield, 1975; Calisher et al., 1989). However, according to the most recent taxonomic classification (Heinz et al., 2000), TBEV belongs to the mammalian group of the tick-borne flavivirus. Other members of the mammalian group include Louping ill virus (LIV), Langat virus (LGTV), Powassan virus (POWV), Omsk hemorrhagic fever virus (OHFV), Kyasanur Forest disease virus (KFDV), Kadam virus (KADV), Royal Farm virus (RFV), Karshi virus, and Gadgets Gully virus (GGYV).

TBEV produces a fatal encephalitis in suckling mice when administered by all routes of inoculation. The pathogenesis of TBEV in laboratory mice has been reviewed by Albrecht (1998). Mice infected orally exhibit meningoencephalitis and shed virus in their feces and milk (Pogodina, 1960). Cows, goats, and sheep experimentally infected by inoculation or tick bite develop viremia and also secrete virus in their milk. This ability of the virus to be secreted in milk and its stability in acidic pH has led to several milk-borne outbreaks of TBE, particularly in Russia, Czech Republic, Austria, and Bulgaria (Gresikova and Calisher, 1988). Many other animals (e.g. rats, guinea pigs, sheep, and swine) are susceptible to infection and develop encephalitis after intracerebral inoculation (Burke and Monath, 2001). Syrian golden hamsters are also susceptible; however, it takes a higher dose of virus to kill these animals than to kill mice. Inapparent infection of adult hamsters was demonstrated by development of complement-fixing antibodies (Slonim et al., 1966a). Rhesus monkeys inoculated intranasally or intracerebrally with the CEE subtype virus develop clinical signs of chronic encephalitis with degenerative spongiform lesions (Zlotnik et al., 1976). In contrast, subcutaneous inoculation of rhesus monkeys leads to a clinically inapparent form with viremia and production of complement-fixing antibodies (Slonim et al., 1966b). Additionally, persistence of TBEV has been demonstrated in rhesus monkeys, as evidenced by virus isolation from monkey tissues by co-cultivation and explantation procedures as late as 383 days after inoculation (Pogodina et al., 1981).

Active immunoprophylaxis against TBEV was first applied in Russia in the early 1940s soon after the recognition of natural foci of virus in that country. A formalin-inactivated vaccine prepared from the brains of virus-infected mice was used in a mass vaccination campaign. The potential for serious allergic reactions to such a vaccine led to the development of a new partially purified vaccine prepared from chick embryo cell cultures, which is currently in use in Russia (Elbert et al., 1985). A purified concentrated inactivated TBE vaccine is also available in Russia and has been shown to provide the same level of protection in experiments with mice as a similar vaccine produced in Austria (Vorob'eva et al., 1996). Furthermore, in a small human clinical trial, the vaccine was highly immunogenic and had low reactogenicity (Chumakov et al., 1991).

In Europe, a partially purified formalin-inactivated TBE vaccine produced in chick embryo cells became available in 1976 (Kunz et al., 1976, 1980). The seed virus used for preparing this vaccine was the Neudoerfl strain of the western subtype isolated from a tick in Austria. This vaccine was highly reactogenetic, producing side effects such as headache, malaise, and fever. These reactions were almost completely eliminated when the vaccine was prepared in a highly purified form by using continuous-flow zonal ultracentrifugation (Heinz et al., 1980) and containing aluminum hydroxide as an adjuvant. This purified vaccine considerably reduced the incidence of TBE, particularly in Austria where, since 1980, 35 million doses of vaccine have been used, 6.8 million people have been vaccinated, and the estimated rate of protection is 96–99% (Gritsun et al., 2003). The highly successful TBE vaccination campaign in Austria resulted in a steady decline of morbidity and an almost complete elimination of disease from that country (Kunz, 2003). In addition to the Austrian vaccine, a second European TBE vaccine is registered in Germany (Klockmann et al., 1989; Harabacz et al., 1992).

Efforts have been made to prepare live-attenuated TBE vaccines from both naturally and experimentally attenuated viruses. In particular, an effort was concentrated on preparing a live vaccine from attenuated Langat virus (Mayer, 1975), but was ultimately unsuccessful. Newer approaches to the development of live-attenuated vaccines are based on the construction of chimeric viruses. A vaccinia virus vector expressing the premembrane (prM) and envelope (E) proteins

induces protective immunity against challenge with virulent TBE virus (Holzer et al., 1999). Many other chimeric virus vaccines are based on the construction of viruses by using infectious clones of non-encephalitic mosquito-borne flaviviruses as a backbone (e.g. yellow fever 17D or dengue type 4 virus). For example, a chimera was created using the dengue 4 virus backbone and containing the prM and E of Langat virus. The chimeric virus was attenuated and protected mice against challenge with highly virulent TBE viruses (Pletnev et al., 2000, 2001). Other experimental approaches are based on vaccination with naked DNA. A naked DNA candidate vaccine expressing the prM and E genes of RSSE and CEE induced protective immunity against RSSE and CEE challenge in mice (Schmaljohn et al., 1997). Follow-up experiments in rhesus macaques showed that this vaccine elicited anti-TBEV antibodies detectable by ELISA and by plaque-reduction neutralization assay (Schmaljohn et al., 1999).

It has been suggested that passive immunization protects against TBE. A specific TBE-immunoglobulin is available in several European countries that can be used for pre- and postexposure prophylaxis. When given within 4 days after tick bite, the protective efficacy was estimated to be 60–70% (Kunz et al., 1981). In a mouse model of TBE, passive immunization with rabbit TBEV antibody resulted in 60% survival when the mice were treated 24 h after viral challenge; however, there was no significant protection if the mice were treated 48 h after viral challenge (Chiba et al., 1999). This work, along with others (Kreil and Eibl, 1997) has suggested that passive protection by immune serum is possible only before infection of the brain is established. However, others reached different conclusions using other flaviviruses in mouse models. For example, protection has been reported even when antibodies were administrated after infection of the brain has been initiated in yellow fever virus and WNV (Camenga et al., 1974; Brandriss et al., 1986). Clearly, more work needs to be done before these issues can be fully resolved. Another area of controversy surrounding passive immunization for TBE is related to antibody-dependent enhancement (ADE). Several case reports suggest that the disease is exacerbated by postexposure passive immunization (Kluger et al., 1995; Arras et al., 1996; Waldvogel et al., 1996). However, other studies demonstrated that in vivo enhancement of TBEV infection by TBEV antibodies could not be observed although those antibodies were able to induce ADE in mouse macrophages in vitro (Kreil and Eibl, 1997). In addition, no indication of ADE was seen in passive immunization experiments in a TBE mouse model (Chiba et al., 1999).

3.2. West Nile virus

WNV, first isolated from the blood of a woman in the West Nile district of Uganda in 1937 (Smithburn et al., 1940), is a member of the Japanese encephalitis virus (JEV) serocomplex that includes other human pathogens such as Japanese encephalitis, Murray Valley encephalitis, St. Louis encephalitis, and Kunjin viruses. After the initial isolation of WNV, the virus was subsequently isolated from patients, birds, and mosquitoes in Egypt in the early 1950s (Melnick et al., 1951; Taylor et al., 1956) and was shown to cause encephalitis in humans and horses. WNV is recognized as the most widespread of the flaviviruses, with a geographical distribution including Africa, the Middle East, western Asia, Europe, and Australia (Hayes, 1989). The virus was first detected in the Western Hemisphere in the summer of 1999, during an outbreak involving humans, horses, and birds in the New York City metropolitan area (CDC, 1999a; Lanciotti et al., 1999). The mechanism by which the virus was introduced into the US may never be known, but studies have shown that the strain isolated during this outbreak (NY-99) had greater than 99.8% nucleotide sequence homology to a strain isolated from the brain of a dead goose in Israel in 1998 (Lanciotti et al., 1999). Additionally, sequence data of reverse-transcriptase polymerase chain reaction products obtained from brain tissue of two human cases of WN encephalitis in Israel also showed a 99.8% homology to the NY-99 strain (Giladi et al., 1999). These data strongly support the hypothesis that the 1999 New York outbreak originated from the introduction of a WNV strain that had been circulating in Israel (Giladi et al., 1999). Since 1999, WNV has extended its range throughout much of the US, and is now considered to be endemic in this country. The extent of spread of WNV into Mexico and Central and South America remains to be seen. Human infections with WNV are generally asymptomatic or produce a mild, undifferentiated fever (West Nile fever), which can last from 3 to 6 days (Monath and Tsai, 2002). In contrast, recent outbreaks of WNV infection in North America, eastern Europe, and Israel are characterized by relatively high rates of fatal neurological disorders (CDC, 1999b, 2001; Hubalek and Halouzka, 1999). The most severe complications are commonly seen in the elderly, with reported case fatality rates from 4 to 11% (Hayes, 1989; Tsai et al., 1998a; Hubalek and Halouzka, 1999; Asnis et al., 2000; Komar, 2000). Severe, non-neurologic manifestations of WNV infection are unusual and include hepatitis, myocarditis, and pancreatitis.

Many early laboratory studies of WN encephalitis were performed in monkeys (Manulidis, 1956; Pogodina et al., 1983) or mice (Eldadah et al., 1967; Weiner et al., 1970). WNV inoculated into monkeys intracerebrally results in the development of overt encephalitis, febrile disease, or an asymptomatic infection, depending on viral strain. The African Eg-101 strain was the most virulent, and experimentally attenuated clones 94 and 98 isolated from the population of the Astrakhan Hp-94 strain were the least virulent for monkeys (Pogodina et al., 1983). In the same study, virus was shown to persist in the brains of experimentally infected rhesus monkeys, regardless of the route of inoculation, for up to 51/2 months (Pogodina et al., 1983). Additionally, virus persistence occurred regardless of the outcome of infection (i.e. asymptomatic, fever, encephalitis).

Thus, virus persistence should be regarded as a typical result of inoculation of nonhuman primates with various WNV strains. After both intracerebral and subcutaneous inoculation, the virus localizes predominantly in the cerebral subcortical ganglia, cerebellum, cerebral cortex, and may also be found in the kidneys, spleen, and lymph nodes. The intracerebrally inoculated monkeys developed a subacute inflammatory-degenerative process in the CNS and this outcome was seen after infection with different strains and clones of WNV that differed in their degree of virulence, antigenic properties, and geographic region of isolation.

Due to the cost and multitude of regulatory issues involved with the use of nonhuman primates in research, most investigators now prefer the less expensive rodent models. All classical laboratory mice strains are susceptible to lethal infections by the intracerebral and intraperitoneal routes resulting in encephalitis and 100% mortality. Recently, Xiao et al. (2001) developed a model for WN encephalitis using the golden hamster, *Mesocricetus auratus*. Hamsters were experimentally infected with the WNV strain NY385-99 isolated from the liver of a snowy owl that died at the Bronx Zoo during the 1999 outbreak in New York City (Steele et al., 2000). Hamsters appeared normal during the first 5 days, became lethargic at approximately day 6, and developed neurologic symptoms at days 7–10. Many of the severely affected animals died 7–14 days after infection. Viremia was detected in the hamsters within 24 h after infection and persisted for 5 or 6 days. Additionally, antibody response, as measured by hemagglutinin inhibition, was detected in the infected animals beginning on day 5. Interestingly, this pattern was the same regardless of the outcome of the infection. Histopathologic examination of hamster organs showed no substantial pathologic changes; however, substantial, progressive pathologic changes were seen in the brain and spinal cord of infected animals. These histopathologic changes in WNV-infected hamsters were similar to those previously reported in parenterally infected adult mice (Eldadah et al., 1967; Weiner et al., 1970). The hamster model appears to more closely approximate human disease than does the mouse model. A comparison of the signs and symptoms of the hamster and mouse models with those seen in WNV-infected patients are listed in Table 2. Furthermore, like the aforementioned monkey experiments by Pogodina et al. (1983), persistent WNV infection was found in the brains of hamsters. Indeed, arboviral persistence seems to be a much more common phenomenon than once thought (Kuno, 2001).

There is no specific antiviral drug treatment for WNV disease. Ribavirin and interferon alpha are active against WNV in vitro (Jordan et al., 2000; Morrey et al., 2002; Anderson and Rahal, 2002), but there are currently no clinical data to support treatment of human disease. Likewise, there is no licensed human vaccine against WNV; however, several laboratories are actively involved in vaccine research. A formalin-inactivated veterinary vaccine produced by Fort Dodge Animal Health (Fort Dodge, IA) was conditionally licensed by the U.S. Department of Agriculture (USDA) in August 2001, and in early 2003 Fort Dodge Animal Health received full-licensed status from the USDA for their product (West Nile-Innovator™). Although this vaccine was protective in a hamster model, two of the nine animals had detectable viremia, suggesting the immune response to the killed vaccine was insufficient to completely inhibit replication of the challenge virus (Tesh et al., 2002). The same company has also initiated development of a DNA plasmid vaccine for horses. The DNA vaccine technology for WNV was developed at the Centers for Disease Control and Prevention, Fort Collins, CO and has protected against viral challenge in mice and horses (Davis et al., 2001). A live attenuated WNV strain was produced by serial passage of a wild-type strain in *Aedes aegypti* mosquito cells and neutralization escape from WNV-specific monoclonal antibody. A single dose of the attenuated virus elicited 100% protection in mice and geese challenged intracerebrally with wild-type virus (Lustig et al., 2000). In addition to work on veterinary vaccines, at least two companies have initiated human vaccine development programs for WNV. Baxter-Immuno in Orth/Donau, Austria has initiated efforts to develop a formalin-inactivated human vaccine, and Acambis Inc. (Cambridge, MA) has developed a live-attenuated vaccine based on its ChimeriVax™ technology, which has also been used in the development of vaccines against Japanese encephalitis and dengue viruses. The ChimeriVax™ technology is based on work by Chambers et al. (1999) and uses yellow fever 17D as a live virus vector. Chambers' chimeric concept originated from earlier work where another pair of flaviviruses, TBEV and dengue, were used to create a chimeric live virus with vaccine potential (Pletnev et al., 1992). In the case of the ChimeriVax™-WN, infectious clone technology was used to replace the genes encoding the prM and E proteins of yellow fever 17D vaccine with the corresponding genes of the WNV. The resulting chimeric virus contains the antigens responsible for protection against WNV, but replicates in the host like yellow fever 17D (Monath, 2001). Using the same technology, Pletnev et al. constructed a chimeric WNV/dengue 4 virus that elicited complete protection to mice challenged by wild-type WNV (Pletnev et al., 2001). The ChimeriVax™-WN also completely protected hamsters from challenge by wild-type virus 1 month after vaccination and induced a strong humoral immune response, suggesting this vaccine will provide long lasting immunity (Tesh et al., 2002). The ChimeriVax™-WN recently completed preclinical studies and will be entering Phase I clinical trails in late 2003.

3.3. Japanese encephalitis virus

JEV is transmitted to humans by *Culex* mosquitoes and is a leading cause of childhood viral encephalitis in southern and eastern Asia. JEV has also been a problem among military personnel and travelers to these regions. It was first

Table 2
Comparison of signs and symptoms of WNV-infected patients and those seen in hamster and mouse animal models[a]

Human subject symptoms[b]	Hamster signs[c], preliminary results[d]	Mouse signs[e], preliminary results[d]
Fever (influenza-like illness, biphasic, chill)	Fever	ND[f]
Abrupt onset (3–6 dpi[g])	Abrupt onset (start at 6 dpi)	Abrupt onset (5 dpi)
Transient viremia (1–10 dpi)	Transient viremia (1–8 dpi)	Transient viremia (1–5 dpi)
Antibody response (start 5 dpi)	Antibody response (start 5 dpi)	Antibody response (start 5 dpi)
Headache often frontal, sore throat	ND	ND
Backpain, myalagia, arthralgia, fatigue	ND	ND
Muscle, motor weakness	Muscle weakness	If present, very short term
Conjunctivitis, retrobulbar pain	ND, exudates from eye socket	ND, exudate not present
Maculopapular or roseolar rash	ND	ND
Lymphadenopathy	ND	ND
Anorexia, nausea, abdomen pain, diarrhea	Diarrhea in some animals	No diarrhea
Respiratory symptoms, short breath	Reduced oxygen saturation	ND
Aseptic meningitis or encephalitis	ND	ND
Neck stiffness, vomiting	ND	ND
Confusion, disturbed consciousness	Balance, circling	Not easily apparent
Somnolence	Somnolence	Somnolence, very short term
Tremor in extremities	Tremor in extremities	Tremors rare
Abnormal relex, convulsion	ND	ND
Altered mental status	ND	ND
Cerebellar abnormality	Cerebellar pathology	Brain pathology
Cranial nerve palsy	ND	ND
Pareses (partial paralysis)	Hind limb paralysis	Paralysis rare
Coma	Unresponsive	Unresponsiveness short term
Death (older patients, <0.1%)	Death (~50%)	80–100%
Elevated cerebrospinal fluid protein	ND	ND

[a] Not all subjects show all symptoms or signs.
[b] Hubalek and Halouzka (1999); Weiss et al. (2001).
[c] Xiao et al. (2001).
[d] Alignment with human symptoms are highly subjective and may not correlate directly.
[e] Haahr (1968, 1971); Katz et al. (2002); Beasley et al. (2002).
[f] ND: not yet determined.
[g] dpi: days postinfection.

isolated from the brain of a patient who died from encephalitis in Japan in 1935 (Burke and Leake, 1988). Later, in 1938, the virus was also isolated from *Culex tritaeniorhynchus* mosquitoes in Japan. *Culex* mosquitoes breed in rice fields and transmit the virus from birds or mammals (mostly domestic pigs) to humans (Hoke et al., 1988).

JEV caused a great epidemic in Japan in 1924, resulting in 6125 cases and 3797 deaths (Burke and Leake, 1988). Although further epidemics occurred in 1935 and 1948, additional epidemics have not been seen in Japan since 1968. JEV caused a major epidemic in Korea in 1949 and in China in 1966. Overall at least 16 countries in eastern, southern, and southeast Asia reported clinical cases of JE in humans (Burke and Leake, 1988).

Disease symptoms vary from a mild febrile illness to acute meningomyeloencephalitis in JEV-infected patients. After an asymptomatic incubation period of 1–2 weeks, patients exhibit signs of fever, headache, stupor, and generalized motor seizures, especially in children. The virus invades and destroys the cortical neurons and causes encephalitis. This neuronal damage is similar to the destruction of anterior horn cells seen in poliomyelitis. The fatality rate ranges from 10 to 50% and most survivors have neurological and psychiatric sequelae (Chen et al., 1999; Guirakhoo et al., 1999).

JEV infection results in fatality in infant mice by all routes of inoculation, and weanling mice are highly susceptible to intracerebral virus inoculation. Differences in pathogenesis are seen when the virus is given by intraperitoneal inoculation (Huang, 1957a,b). These differences depend on the amount of virus and the specific viral strains used. Mice show biphasic viral multiplication in their tissues after peripheral inoculation. The early-primary viral replication occurs in the peripheral tissues and the later-secondary phase in the brain (Huang and Wong, 1963). Studies also demonstrate that pregnant mice inoculated with JEV intraperitoneally transmit the virus to the fetus and this transmission significantly increases the incidence of abortion (Mathur et al., 1982).

Severe histopathological changes are observed in brain hemispheres including substantia nigra, thalamus, and lenticulo-striate complex when rhesus monkeys are inoculated intracerebrally with JEV. Symptoms such as weakness, tremors, and convulsions began to appear on days 6–10, then death, preceded by clear signs of encephalomyelitis, occurred on days 8–12 postinfection for most of the animals (Nathanson et al., 1966). Intranasal inoculation of JEV also results in fatality in both rhesus and cynomolgus monkeys, but asymptomatic viremia was observed after peripheral

inoculation (Harrington et al., 1997; Burke and Monath, 2001).

Intracerebral or intranasal inoculation of JEV killed hamsters while peripheral inoculation caused asymptomatic viremia. Studies with rabbits and guinea pigs showed that all routes of inoculation of JEV produce asymptomatic infection in these animals. While cattle are not affected by JEV, horses and swine are susceptible to infection with the virus (Burke and Monath, 2001).

Currently, three JEV vaccines are in use (Guirakhoo et al., 1999; Chang et al., 2000): (i) an inactivated vaccine derived from mouse brains is the only vaccine currently in use internationally; (ii) a cell culture-derived inactivated vaccine; (iii) a cell culture-derived live attenuated vaccine in use only in China. Although the formalin-inactivated, mouse brain-derived vaccine is safe and effective, it is very expensive for routine vaccination in most Asian countries. A live attenuated vaccine has been used only in China. Due to various regulatory issues, this vaccine is not used outside of China.

During World War II, inactivated JEV vaccines prepared from suspensions of infected mouse brains were given to US soldiers (Hoke et al., 1988). This unpurified vaccine was tested in Taiwan in 1965 and was shown to be effective. Although this vaccine appeared to be efficacious, adverse reactions such as hypersensitivity reactions consisting of generalized urticeria and angioedema forced the development of improved vaccines (Tsai et al., 1998b). In 1965 a highly purified JEV vaccine, which is a formalin-inactivated preparation purified from infected mouse brains, was developed by the Research Foundation for Microbial Disease of Osaka University (Biken) in Japan using the Nakayama strain of JEV. The Nakayama strain of JEV was isolated from the cerebrospinal fluid (CSF) of a patient in 1935 and maintained by continuous mouse brain passage and has been the principal strain used in mouse brain-derived vaccines produced throughout Asia (Oya, 1988). This vaccine was used for routine vaccination of children in Japan for many years, although its efficacy was never tested. In 1982, the inactivated JEV vaccine was licensed as JE-VAX in the US, Canada, Israel, and several Asian countries. The vaccine is given subcutaneously in two doses 1–4 weeks apart, with a booster dose at 1 year and additional booster doses at 1–3 years thereafter. Due to the natural diversity of JEV strains, a second mouse brain-derived vaccine was produced based on the Beijing-1 strain (also known as the P1 in China or the equivalent P3). The Beijing-1 strain grows to higher titer and the vaccine produces higher heterologous antibody titers in vaccinated mice than does the Nakayama strain vaccine (Tsai et al., 1999). Biken, the principal Japanese manufacturer of JE vaccine, has used the Beijing-1 strain since 1989 in vaccine produced for domestic consumption, whereas the Nakayama strain is used in vaccines distributed internationally (Tsai et al., 1999). Later, a cell culture-derived inactivated vaccine containing the P3 strain was developed. The P3 strain of JEV was recovered in 1949 from the brain of a patient during the Beijing-1 (P1) strain epidemic. This vaccine, prepared in primary hamster kidney cells, is produced exclusively in China and has been that country's principal JEV vaccine since 1968. The vaccine is given seasonally in early spring just before the JEV transmission season. Vaccination schedules vary locally, but in the recommended schedule, the vaccine is given subcutaneously in two doses, 1 week apart, to children 12 months old. Booster doses are given 1, 6, and 10 years later (Tsai et al., 1999). In certain provinces, where JE cases were occurring in younger children, primary immunization with two doses was begun at 6 months of age and was shown to provide 85% protection in infants after primary immunization.

A live attenuated JEV vaccine based on a stable neuroattenuated strain of the JEV (SA14-14-2 strain) was produced in primary hamster kidney cells and was licensed for use in China in 1988 (Tsai et al., 1998b). Currently, over 30 million doses are distributed annually in several southwestern provinces and selected regions of China and is being used for routine vaccination of children in that country (Tsai et al., 1998b). Two primary doses given at intervals of 1 or 2.5 months were shown to produce immunity in 94–100% of vaccinated school-aged children (Tsai et al., 1998b). A case-controlled study in rural Sichuan Province, China, concluded that a regimen of two doses administered 1 year apart was to prevent clinically important disease (Hennessy et al., 1996).

DNA vaccines also provide protection against JEV infection. Vaccination with plasmid DNA (pCMXENV) expressing JEV E protein provides significant protection against intracerebral viral challenge with JEV (Ashok and Rangarajan, 2000). Although JEV-specific antibodies were not detected in mice inoculated intramuscularly or intranasally pCMXENV, an increase in JEV-specific T cells, enhanced production of interferon-gamma, and complete absence of interleukin-4 were observed after JEV challenge. These results indicate that protection is most likely mediated by T helper lymphocytes of the Th1 sub-type (Ashok and Rangarajan, 2000). It was also reported that DNA vaccines containing the JEV E gene (Chen et al., 1999) or JEV E and prM genes (Konishi et al., 1998a) were able to provide protection against a lethal JEV challenge.

Second generation-recombinant JEV vaccines have focused on expression of the relevant immunogens in adenoviruses, vaccinia viruses, or baculoviruses (Konishi et al., 1997). Infectious vaccines consist of attenuated viral isolates generated from infectious cDNA clones, while noninfectious vaccines contain immunogen JEV proteins (Konishi et al., 1997). Three JEV proteins, prM, E, and nonstructural (NS) proteins, used in non-infectious vaccines are glycosylated and capable of inducing protective immunity. Recombinant JEV vaccine consisting of genes extending from prM to nonstructural 2B (NS2B) in a vaccinia virus backbone protected challenged mice (Konishi et al., 1991, 1992). Moreover, recombinant vaccinia viruses expressing the prM, E, and NS1 genes of JEV based on the highly attenuated

vaccinia virus strain (NYVAC-JEV) or host range restricted canarypox virus (ALVAC-JEV) were tested in phase I human trials and only 1 in 10 ALVAC-JEV vaccinated individuals developed detectable viral neutralizing antibody against JEV (Konishi et al., 1998b). Studies with rhesus monkeys indicated that NYVAC-JEV and ALVAC-JEV vaccines are safe and effective in protecting monkeys from JE while ALVAC-JEV vaccine showed less immunogenicity in monkeys than NYVAC-JEV vaccine (Kaengsakulrach et al., 1999). Expression of the prM and E proteins of JEV by a recombinant vaccinia virus vector produced extracellular subviral particles. Konishi et al. (2001) demonstrated that these extracellular particles contained the JEV prM and E proteins, which were highly immunogenic in mice and induced neutralizing antibodies, virus-specific cytotoxic T lymphocytes, and resulted in protective immunity.

Recently, the World Health Organization (WHO) made the development of new JEV vaccines a high priority for further research (Chambers et al., 1997). In 1999, construction of JEV and yellow fever chimeric virus containing the core and NS genes of the yellow fever vaccine strain (YF-17D) and prM and E genes of the attenuated JEV strain SA14-14-2 strain increased the hope to develop safe, effective and single-dose JEV vaccines (Chambers et al., 1999). ChimeriVax-JE was shown to be safe in mice and monkeys, and a single dose inoculated subcutaneously in mice protected them from intraperitoneal challenge with a virulent JEV (Guirakhoo et al., 1999). Vaccine studies in monkeys demonstrated similar results when they were challenged intracerebrally (Monath et al., 1999). Phase I trials with 12 human volunteers indicated the safety and immunogenicity of the vaccine, and a Phase II challenge trial was successfully completed in 2001 for ChimeriVax-JE vaccine (Monath et al., 2002).

3.4. St. Louis encephalitis virus

St. Louis encephalitis virus (SLEV) was first isolated during a large epidemic in St. Louis and Kansas City, Missouri in 1933 (Chamberlain et al., 1957). The disease was characterized by variable severity of CNS infections. During the 1940s, the virus spread to the Pacific coast of the US and then, between 1959 and 1961 to south Florida. Several outbreaks have been observed in the western US, Texas, Ohio-Mississippi River valleys, and Florida since the first epidemic in Missouri (Tsai et al., 1986; Monath and Tsai, 1987). Several species of *Culex* mosquitoes are major vectors of the virus (Burke and Monath, 2001). SLEV infection can result in symptoms ranging from febrile headache to encephalitis. Severe disease and encephalitis is most often observed in the elderly. After a 4–21-day incubation period, malaise, fever, chilliness, headache, drowsiness, anorexia, nausea, myalgia, and sore throat as well as meningeal and neurological signs are observed in infected patients. It mainly affects the substantia nigra, thalamus, and hypothalamus of the CNS and causes neuronophagia, cellular nodules, and perivascular cuffing. Elderly patients experience a 22% fatality. It has been reported that chronic disease or immune suppression increases the risk of developing encephalitis (Okhuysen et al., 1993).

Laboratory animals such as mice, monkeys, rats, chickens, guinea pigs, and rabbits have been used to study the pathogenesis of SLEV infection. The susceptibility of mice to SLEV infection varies with viral strain as well as route of infection. Similarly, infection with different strains of SLEV results in varying pathogenesis in rhesus monkeys inoculated intracerebrally (Monath et al., 1980).

Currently, there are no licensed SLEV vaccines available for human use. After World War II, a relatively effective inactivated mouse brain-derived vaccine was prepared using the Webster strain of SLEV (Sabin et al., 1943); however, this vaccine was never mass-produced or field-tested. The possibility of potential side effects associated with the mouse brain-derived vaccine forced the development of new vaccine alternatives against SLEV infection. A DNA vaccine consisting of a plasmid encoding the prM and E proteins of SLEV provided partial protection (25% of the animals survived) from lethal viral challenge, but no neutralizing antibodies were detected in mice inoculated twice with the vaccine (Phillpotts et al., 1996). It has also been demonstrated that vaccination with recombinant baculovirus expressing the same proteins elicited high levels of neutralizing and protective immune responses in mice (Venugopal et al., 1995).

3.5. Murray Valley encephalitis virus

Although Murray Valley encephalitis virus (MVEV) was first recognized as an infectious agent in 1917, the virus itself was not characterized at that time. It was not until the 1951 outbreak that the virus was first isolated from the brain of an infected human (French, 1952). Most of the MVEV epidemics occurred in the Murray Valley region of New South Wales and Victoria during the summer; however, sporadic cases were also observed in New Guinea (Marshall, 1988). *Culex annulirostris* is the primary vector for this virus (Doherty et al., 1963). The virus has also been isolated from *Aedes normanensis* and several *Culex* species mosquitoes. After the incubation period, fever, headache, myalgia, anorexia, nausea, and neurological signs can be observed in infected patients. Symptoms can vary from mild disease with altered level of consciousness to severe CNS damage and death. While only fewer than 1% of infected individuals develop encephalitis, infection can result in 25% fatality with neurological sequelae in 50% of patients (Broom et al., 2000).

MVEV infects newborn mice via all routes of inoculation. Although, susceptibility of mice decreases in mice 17–28 days of age via peripheral routes, they are still susceptible to infection via intracerebral inoculation (French, 1973). Varying severity of CNS disease in mice is directly related to the particular strain of MVEV used. Challenge with a virulent, highly invasive strain quickly spreads through lymph nodes

and produces viremia. The virus then reaches the CNS through the olfactory bulb. Flicking of the ears, tonic and clonic spasms, and lack of coordination are the first symptoms seen in MVEV-infected mice. Some mice may have tremors, much like those seen in patients with Parkinson's disease. Newborn mice die from encephalitis 5–7 days postinoculation (French, 1952). MVEV infection results in fatality in 6–10-week-old hamsters via all routes of inoculation. Intracerebral inoculation of MVEV causes encephalitis in some birds, sheep, horses, and monkeys. Chickens develop encephalitis resulting in fatal disease 4–6 days post intracerebral or intramuscular inoculation of MVEV. Rabbits and guinea pigs develop subclinical disease with low viremia via intracerebral and peripheral inoculations and all animals develop complement-fixing antibodies against MVEV in approximately 3 weeks after inoculation (French, 1952, 1973).

Presently, there are no approved vaccines available for MVEV infection; however, many efforts are underway. DNA vaccines and a Semliki Forest virus-vectored vaccine using prM and E proteins is under investigation for future human use (McMinn et al., 1996; Colombage et al., 1998). Furthermore, subviral particles produced by the in vitro expression of recombinant MVEV prM and E genes prevent mice from developing encephalitis (Kroeger and McMinn, 2002).

3.6. Other flaviviruses causing encephalitis

Rocio virus was first isolated from fatal human cases in 1975 from a large outbreak of encephalitis on the south coast of São Paulo State, Brazil (de Souza Lopes et al., 1978a,b). The virus serologically cross-reacts most closely with members of the JEV serocomplex, and confirmation of this grouping has been reported with recent molecular phylogenetic analysis (Batista et al., 2001). Rocio virus causes encephalitis and death in suckling and weaned mice inoculated intracerebrally and intraperitoneally. Suckling hamsters inoculated intracerebrally also developed fatal encephalitis (de Souza Lopes et al., 1978a). In a separate study, experimentally infected suckling hamsters developed severe necrosis of the myocardium and pancreas (Harrison et al., 1980). In humans, the disease is similar to that seen with JEV and SLEV; the case fatality rate is approximately 4%, and sequelae, most notable, persistent cerebellar, motor, and neuropsychiatric signs, occurred in 20% of survivors (Burke and Monath, 2001). Shortly after the initial outbreak in Brazil, a formalin-inactivated vaccine was prepared from infected suckling mouse brains. A pilot study was conducted at a site near the epidemic area; however, the vaccine lacked potency (de Souza Lopes et al., 1983).

In addition to TBEV, other members of the mammalian group of tick-borne flaviviruses are known to cause encephalitis in humans, including louping ill virus (LIV) and Powassan virus (POWV). These viruses do not produce significant epidemic outbreaks in humans and, therefore, are considered less important than TBEV. LIV is closely related to TBEV and occurs in Scotland, England, Wales, and Ireland and primarily causes a neurologic disease (louping ill) in sheep (Reid, 1988). A formalin-inactivated vaccine protects sheep, and has been used to a limited extent in humans. More recently, a recombinant Semliki Forest virus vaccine, encoding the prM/E and NS1 proteins of LIV completely protected sheep against a subcutaneous virus challenge (Morris-Downes et al., 2001).

POWV was first isolated from the brain of a 5-year-old boy who developed encephalitis and died in 1958 in Ontario, Canada (Artsob, 1988). POWV is now known to circulate in the US, Canada, and eastern Russia, and possibly other areas, including China and Southeast Asia (Hoogstraal, 1981). In the US and Canada, POWV causes severe encephalitis in humans with a high incidence of neurological sequelae and up to 60% case fatality rate. The virus is pathogenic for infant and weanling mice by the intracerebral and intraperitoneal routes; and hamsters and rabbits develop subclinical infections. Experimental encephalitis has been demonstrated in rhesus macaques. Pathologic changes in the brains of mice, monkeys, and humans are typical of other flavivirus infections. There is no available vaccine for POWV. Additionally, vaccination against TBEV produces only low-titer cross-reactive antibodies that are not considered sufficient for protection against POWV.

Recently, a new TBE-like virus was isolated from *Ixodes scapularis* ticks collected from sites in coastal New England (Telford et al., 1997). The infection rate among adult *I. scapularis* was similar to those reported for enzootic TBEV in Europe. An enzootic transmission of the virus, referred to as deer tick virus (DTV), was also identified in northern Wisconsin (Ebel et al., 1999). DTV is similar to, but distinct from, POWV and may represent a new subtype of POWV. To date, there have been no known human cases of DTV infection and the public health significance of DTV remains unknown.

4. Togaviridae

Members of the *Alphavirus* genus of the family Togaviridae are mosquito-borne viruses that compose an important group of disease agents (Calisher et al., 1980; Griffin, 1986). The New World alphaviruses include western equine encephalitis virus (WEEV) and eastern equine encephalitis virus (EEEV), both of which can cause severe disease in horses and encephalitis in humans. WEEV has a wide geographic range, and is found from western Canada to Mexico and, discontinuously, to Argentina. WEEV is transmitted in the western US by the mosquito *Culex tarsalis* and birds serve as the vertebrate reservoir. EEEV can be found in the eastern US and its primary vector is *Culiseta melanura*. Another New World virus is Venezuelan equine encephalitis virus (VEEV), which is found in Central and South America (Hahn et al., 1988). Several species of mosquitoes are responsible for the transmission of VEEV and account for either the enzootic or epizootic cycles of the virus.

Human outbreaks of all three of these viral diseases occur shortly after outbreaks are observed in horses. EEEV is the most virulent of these encephalitic alphaviruses, resulting in high mortality due to encephalitis. Although WEEV can also cause human disease, fatal encephalitis is not as great as that associated with EEEV. Finally, VEEV can cause significant disease in humans, with transmission occurring by the respiratory route as well as by mosquitoes (Griffin, 2001). This former route of infection is primarily associated with laboratory workers and accounts for the eighth highest laboratory-acquired infection reported to the Centers for Disease Control and Prevention.

Although human cases of WEE and EEE combined in the US have numbered less than 1000 since 1964, both are considered to be emerging infectious diseases. This designation is primarily due to environmental changes associated with movement of humans into previously undeveloped areas where the virus lives and the expansion of agricultural irrigation, which has created a favorable habitat for mosquito vectors and bird reservoirs. Conversely, outbreaks of VEEV are more common and pose a substantial risk for humans. Therefore, the development of licensed, efficacious vaccines against these viruses is needed. Currently, there are vaccines available under Investigational New Drug (IND) status for all three viral diseases. However, these vaccines were developed over 30 years ago and each has associated side effects and disadvantages. Several promising candidate vaccine constructs have been developed for VEEV, but new candidate vaccines for EEEV and WEEV are limited.

4.1. Venezuelan equine encephalitis virus

VEEV is maintained in nature in a cycle between small rodents and mosquitoes (Johnstone and Peters, 1995). Spread of epizootic strains of the virus (serogroup 1, variants A/B and 1C) to equines leads to a high viremia followed by a lethal encephalitis, and tangential spread to humans. Furthermore, VEEV can easily be spread by aerosol infection, making it a considerable laboratory hazard and a potential bioweapon. During an epidemic in the mid-1990s in Colombia, an estimated 8% of the country's equines may have died and there were an estimated 75,000 human cases associated with the epidemic (Rivas et al., 1997).

Laboratory animals such as mice, guinea pigs and monkeys exhibit different pathologic responses when infected with VEEV. While VEEV infection in guinea pigs and hamsters produces a very short, acute, febrile disease without signs of CNS disease, it causes paralytic disease in mice. Guinea pigs and hamsters generally die within 2–4 days after infection and fatality is not dose-dependent (Berge and Gochenour, 1958; Gleiser et al., 1961; Gorelkin and Jahrling, 1975). Disease caused by VEEV infection lasts longer in mice which show the signs of nervous system disease in 5–6 days and death 1–2 days later. In contrast to guinea pigs and hamsters, the time to death in mice is dose-dependent (Berge and Gochenour, 1958; Gleiser et al., 1961).

Studies with monkeys show that VEEV infection causes a typical biphasic febrile response. Initial fever was observed at 12–72 h after infection and lasted less than 12 h. Then secondary fever generally began on day 5 and lasted 3–4 days (Berge and Gochenour, 1958; Gleiser et al., 1961). Leukopenia was common in animals exhibiting fever (Monath et al., 1974). Mild symptoms such as anorexia, irritability, diarrhea, and tremors also have been observed in VEEV-infected monkeys (Gleiser et al., 1961; Monath et al., 1974). Furthermore, microscopic changes in lymphatic tissues such as early destruction of lymphocytes in lymph nodes and spleen, a mild lymphocytic infiltrate in the hepatic triads, focal myocardial necrosis with lymphocytic infiltration have been observed in monkeys infected with VEEV (Gleiser et al., 1961; Monath et al., 1974). Characteristic lesions of the CNS were observed in monkeys in spite of the lack of any clinical signs of infection (Gleiser et al., 1961). The primary lesions were lymphocytic perivascular cuffing and glial proliferation and generally observed at day 6 postinfection during the secondary febrile episode.

The lymphatic system is a general target in all animals infected with VEEV. However, CNS involvement was variable among different animal species. Histopathologic studies showed massive necrosis of lymphocytes in lymph nodes, spleen, and necrosis and depopulation of bone marrow, as early as day 2 in guinea pigs and hamsters. In addition to lymphocytic destruction, encephalomyelitis was clearly observed in mice (Gleiser et al., 1961).

Subcutaneous infection in the mouse model results in encephalitic disease very similar to that seen in horses and humans (MacDonald and Johnson, 2000). Virus begins to replicate in the draining lymph nodes at 4 h postinoculation. Eventually, virus enters the brain primarily through the olfactory system. Furthermore, aerosol exposure of mice to VEEV can result in massive infection of the olfactory neuroepithelium, olfactory nerves, and olfactory bulbs and viral spread to brain, resulting in necrotizing panencephalitis (Charles et al., 1995; Steele et al., 1998). The clinical signs of disease in mice infected by aerosol are ruffled fur, lethargy, and hunching, progressing to death (Steele et al., 1998).

Animals surviving longer such as horses and monkeys show regenerate lymphatic and hematopoietic activity and develop subsequent encephalitic signs. Hamsters, however, demonstrate very few regenerative changes and die long before encephalitis is observed (Gorelkin and Jahrling, 1975). In contrast to other species, lymphoreticular and myeloid tissue destruction is the primary pathology observed in hamsters, guinea pigs, and rabbits infected with VEEV (Victor et al., 1956; Walker et al., 1976).

Enzootic strains of the virus occur primarily in subtropical and tropical areas of the Americas. Humans living in these areas have a high prevalence of antibody, but little recognized disease (Grayson and Galindo, 1968; Scherer et al., 1972). Epizootic strains of the virus cause epidemic outbreaks of the disease in 10- to 20-year intervals, especially in the ranch areas of Venezuela, Colombia, Peru, and

Ecuador, when heavy rains lead to increased populations of *Aedes taeniorhynchus* and *Psorophora confinnis* (Rivas et al., 1997). In humans, infection with VEEV causes a sudden onset of malaise, fever, chills, headache, and sore throat (Johnson et al., 1968; Johnson and Martin, 1974; Peters and Dalrymple, 1990). Symptoms persist for 4–6 days, followed by a 2–3-week period of generalized weakness. Encephalitis occurs in a small percentage of adults ($\leq$0.5%); however, the rate in children may be as high as 4%. Neurologic symptoms range from mild cases of nausea, vomiting with a decreased sensorium, nuchal rigidity, ataxia, and convulsions to the more severe cases exhibiting coma and paralysis (Johnson et al., 1968; Peters and Dalrymple, 1990). The overall mortality rate in humans is <1% (Pittman et al., 1996). Thus, an efficacious vaccine for human use would be of great benefit in eliminating human disease associated with VEEV.

In 1961, the U.S. Army developed a live, attenuated VEE vaccine, TC-83 (Berge et al., 1961). This vaccine was prepared by serial passage of the Trinidad strain of the virus in fetal guinea pig heart cells. This vaccine has U.S. Food and Drug Administration (FDA) IND status for use in humans and has been used to protect at-risk laboratory workers against infections. However, this vaccine is responsible for some serious side effects in humans. For example, a high rate of reactogenicity (25%), including systemic febrile illness, has been reported (McKinney, 1972). Up to 20% of those individuals vaccinated failed to develop neutralizing antibody titers (Pittman et al., 1996) and some vaccinees that do initially respond to the vaccine do not develop a significant immune response when boosted by re-vaccination with TC-83 once titers have dropped below acceptable values. Furthermore, there have been suggestions of the potential abortogenic and teratogenic effects of the vaccine (Berge et al., 1961; McKinney et al., 1963; Johnson et al., 1968; Casamassima et al., 1987; Peters and Dalrymple, 1990). These findings prompted the development of alternative vaccines.

The first alternative vaccine for VEEV was developed in 1974. This vaccine, called C-84, is a formalin-inactivated VEEV harvested from the TC-83 vaccine production seed, which was passaged twice in chick embryo cells (Cole et al., 1974). Evaluations of this vaccine as both a primary and booster in humans revealed only mild local and systemic reactions (Edelman et al., 1979).

A study performed at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) in a total of 821 personnel investigated clinical reactions to TC-83 and C-84 as well as the ability of vaccines to produce neutralizing antibodies. Of the personnel involved in this study, 128 were vaccinated with the formalin-inactivated C-84 vaccine. These individuals had previously received the live, attenuated TC-83 vaccine and did not respond with an adequate immune response or developed an immune response which fell below the acceptable VEEV 80% plaque reduction-neutralization titer ($PRNT_{80}$) of 1:20 and required a booster. Responses of individuals receiving the live attenuated TC-83 vaccine were modest with a geometric mean titer of 1:83. Neutralizing antibody titers were detected in 89% of vaccine recipients. The use of C-84 has been less extensive than that of TC-83. Results of this study demonstrated that the use of C-84 as a booster immunogen provides excellent antigenic recall with a four-fold rise in antibody titers. Some individuals in this study failed to respond to either vaccine preparation. This could reflect the absence of proper processing mechanisms of major histocompatability complex (MHC) molecules for proper viral antigen presentation, lack of an appropriate helper T-cell subset, presence of antigen-specific suppressor cells, or some other immunoregulatory mechanism, such as cytokine release (Pittman et al., 1996).

4.1.1. Live recombinant vaccines

With the successful eradication of smallpox, investigators have looked to vaccinia virus as a possible tool in the development of a recombinant vaccine vector (Tartiglia et al., 1990). The first use of vaccinia virus to generate a recombinant viral vaccine against VEEV expressed cDNA encoding the structural proteins of the TC-83 vaccine strain. This recombinant strain was designated TC-5A, and this vaccine was quite successful in protecting mice and horses from an intraperitoneal challenge with virulent VEEV, but was unable to protect mice against an intranasal challenge (Kinney et al., 1988; Bowen et al., 1992). Further studies with this vaccine construct in non-human primates elicited reduced immune responses as well as reduced protection when compared to TC-83 (Monath et al., 1992). However, a recombinant vaccinia virus vaccine similar to TC-5A was constructed by other investigators and was designated WR100. These investigators also made an additional construct, which contained a synthetic vaccinia promoter to increase the production of VEEV proteins. Furthermore, the amino acid sequence of the E2 glycoprotein was modified to improve immunogenicity; this vaccine construct was designated WR103. Studies comparing the TC-83 vaccine to these recombinant vaccinia constructs demonstrated that although the WR103 construct was able to provide some protection to mice infected subcutaneously with virulent VEE Trinidad strain, its level of protection was less than that afforded by TC-83 vaccine. The WR100 construct provided minimal protection from challenge. Differences in survival observed between these recombinant constructs are postulated to be attributed to the E2 epitope alteration in WR103, allowing for a higher level of VEE protein expression.

When comparing antibody response of WR100 and WR103 to TC-83, it was determined that the WR103 vaccine elicited a detectable amount of anti-VEE antibody, although it was substantially lower than that found in mice vaccinated with TC-83. Furthermore, mice vaccinated with WR100 failed to make a detectable amount of antibody. Similarly, neutralizing antibody was found in serum from mice vaccine with TC-83, but were not detected in mice vaccinated with the recombinant constructs (Bennett et al., 1998).

4.1.2. Monoclonal antibody-based vaccines

Due to the logistical impracticality of mass vaccination of people exposed to VEEV during an epizootic, the use of monoclonal antibodies (Mabs) has also been investigated as a possible antiviral therapy. In recent years, the use of Mabs has found increased applications for antiviral therapy (Krause et al., 1997; Weltzin and Monath, 1999; Zeitlin et al., 1999). Two Mabs that demonstrated potent protective activity against a subcutaneous VEEV challenge in mice were subsequently tested in mice challenged by aerosol with VEEV (Phillpotts et al., 2002). These Mabs were administered 24 h before challenge and provided excellent protection (90–100%) from a virulent challenge of 100 LD_{50} of virus. When the Mabs were administered 2 or 24 h after aerosol challenge, survival dropped to 50%. Although survival was lower when the Mabs were administered after challenge as compared to before challenge, this was a significant observation. This demonstrated that Mab treatment may have a beneficial effect upon existing VEEV infection, but indicated the Mab must be given early in the course of infection. The mechanism by which the Mab therapy provides protection from challenge was also investigated. Investigators determined that Mabs might be able to abort infection in some mice and to prevent the spread of virus to the brain in others. Because antibody to VEEV appears in the circulation soon after infection (Pederson and Eddy, 1974; Johnson and Martin, 1974) the Mab treatment may delay viral replication, thus giving the host immune response time to respond and control viral infection.

4.1.3. Live, attenuated vaccines

The live attenuated vaccine candidate V3526 is a molecularly defined vaccine. Attenuation was achieved by combining the deletion of the four-amino acid furin cleavage sequence in the PE2 glycoprotein and an amino acid change in the E1 glycoprotein residue 253 (Schmaljohn et al., 1982). The V3526 vaccine was administered subcutaneously and by aerosol. Both routes completely protected mice challenged subcutaneously with virulent VEEV. Analysis of the immune response indicated that V3526 induced high titers of virus-neutralizing antibodies equivalent to those induced by TC-83. Furthermore, all mice vaccinated subcutaneously with V3526 seroconverted to the vaccine, indicating it was more effective that TC-83 for inducing a murine immune response, as some 12% of mice vaccinated with TC-83 do not seroconvert. Immunological studies indicate that the V3526 vaccine induced both systemic and mucosal immunity and indicates that both arms of the immune system play a significant role in providing protection from virulent challenge. Furthermore, the ability of the V3526 vaccine to protect mice against a subcutaneous challenge when vaccinated via the aerosol route, makes this vaccine an excellent candidate for future development of a nasal delivery system (Hart et al., 2000).

Another live, attenuated candidate vaccine construct is V3014. It contains two mutations in the surface glycoproteins, which confer its attenuating phenotype. Mice vaccinated subcutaneously with a single dose of V3014 and subsequently challenged with virulent VEEV either via the intranasal or intraperitoneal routes, all survived challenge regardless of the route of challenge, and none exhibited any clinical signs of illness. Furthermore, the IgG antibody response to V3014 was quite high and remained so for at least 7 weeks. Further investigation of this vaccine indicated that the immunizing virus invades and replicates within the Peyer's patch, perirectal, and submandibular lymph nodes making it likely that parenteral inoculation with V3014 stimulates mucosal immunity. Targeting of this attenuated VEE vaccine to mucosal associated lymphoid tissue suggests that this type of live, attenuated virus may also serve as an excellent vaccine expression system for vaccination against mucosal pathogens (Charles et al., 1997).

4.1.4. Microencapsulation

Microspheres have been shown to be an effective vehicle and adjuvant for potentiating immune responses when administered by either the parenteral or mucosal route (Greenway et al., 1995). The use of microcapsules containing VEEV to augment protective immunity has also been investigated as a potential alternative vaccine for VEEV. Formalin-fixed or untreated, inactivated TC-83 virus was microencapsulated in poly (D,L-lactide-co-glycolide) microspheres, made either with methylene chloride or ethyl acetate. Mice were vaccinated twice subcutaneously with various amounts of the microencapsulated TC-83 preparation and subsequently challenged intraperitoneally with virulent VEEV. Microencapsulation of VEEV was able to provide 100% protection to mice after challenge. This level of protection was identical for both preparations of the microcapsules. All doses and preparations of the microcapsules induced higher antibody responses than those observed in mice given the TC-83 vaccine. Furthermore, the microencapsulated VEEV vaccine induced systemic immune responses, which were higher and persisted longer than those induced with the free virus. When considering the amount of antigen present in the microencapsulated vaccines, the methylene chloride preparations at the lower antigen concentration elicited higher antibody responses after both the primary and secondary vaccination. This immune response persisted in the mice given the vaccine prepared with methylene chloride, but waned in those vaccines prepared with ethyl chloride. Furthermore, the methylene chloride vaccine preparations were superior in inducing a virus-neutralizing antibody titer (Greenway et al., 1995).

These studies also determined that a higher IgG anti-VEEV response and higher neutralizing titers were achieved when the TC-83 vaccine was formalin fixed before microencapsulation. This suggests that the formalin fixation of the whole virus was important in preserving antigenic epitopes during the microencapsulation process (Greenway et al., 1995).

4.2. Eastern equine encephalitis virus

EEEV was first recognized as a disease of horses in the northeastern US in 1831. More than 75 horses died in three counties along the coast of Massachusetts during the summer of 1831. Furthermore, epizootics were recorded between 1845 and 1912 in New York, North Carolina, New Jersey, Maryland, and Virginia (Hanson, 1957; Scott and Weaver, 1989). However, it was not until 1933 that the virus responsible for EEE was isolated from the brains of infected horses. Epizootics occur approximately every 5–10 years, and are associated with heavy rainfall, and warm water temperatures that increase the population of mosquito vectors (Grady et al., 1978; Morris, 1988; Letson et al., 1993; Mahmood and Crans, 1998; Takeda et al., in press). The largest recorded outbreak of EEEV occurred in 1947 in Louisiana and Texas, with 14,344 cases of equine encephalitis and 11,722 horse deaths (Chang and Trent, 1987). The association of human disease with epizootics had been suspected, but not until the outbreak in 1938 had a link been confirmed. Thirty cases of fatal encephalitis in children living in the same area as the equine cases provided this evidentiary link between equine and human disease. EEEV was isolated from the CNS of these children as well as from pigeons and pheasants (Morris, 1988).

EEEV can cause localized outbreaks of disease in the summer, primarily in areas near salty marshes. The virus is enzootic from the coastal areas of New Hampshire southward along the Atlantic seaboard and westward to the Gulf coast in Texas and continuing south into the Caribbean and Central America. Occasional inland foci of the virus occur and have been found in the Great Lakes regions and South Dakota (Morris, 1988). EEEV is also enzootic along the coasts of South America and in the Amazon Basin; however, human infections in these regions cause only mild or subclinical disease (Causey et al., 1961). Regardless of where the virus is found, the enzootic cycles are maintained in moist environments; shaded marshy salt swamps in North America and moist forests in Central and South America (Griffin, 2001). The virus is transmitted by mosquitoes, and birds appear to be the primary reservoir host. *C. melanura* is the primary enzootic vector for EEEV in North America. Many species of birds are susceptible to infection but remain asymptomatic despite prolonged viremia (Kissling et al., 1954). In North America, wading birds, migratory passerine songbirds, and starlings are primarily responsible for virus amplification (Dalrymple et al., 1972; McLean et al., 1995; Komar et al., 1999). Young birds, in particular, are important in virus amplification due to their increased susceptibility to infection, prolonged viremia, and their less defensive nature toward mosquitoes (Dalrymple et al., 1972). Interestingly, in Central and South America, forest-dwelling rodents, bats and marsupials frequently become infected and may provide an additional reservoir. However, transmission cycles in these animals are not well characterized (Scott and Weaver, 1989; Ubico and McLean, 1995). Reptiles and amphibians have also been reported to become infected (Morris et al., 1997).

Outbreaks of EEEV occur primarily in summer months and the mechanism enabling the virus to survive winter in temperate areas is not known. It is postulated that the virus is maintained by resident birds or is reintroduced annually by migratory viremic birds or wind-borne infected mosquitoes coming from sub-tropical areas where transmission occurs year round (Sellers and Maarouf, 1990; Weaver et al., 1999).

Antigenic differences between strains of EEEV isolated in North and South America have long been recognized (Casals, 1964; Calisher et al., 1971). The strains can easily be distinguished based upon reactivity to the E1 glycoprotein with Mabs (Roehrig et al., 1990). At the present time, one strain of EEEV is recognized in North America and the Caribbean and three strains are found throughout Central and South America (Brault et al., 1999; Weaver et al., 1999).

EEEV pathogenesis and disease has been studied in several laboratory animals. Susceptibility to EEEV varies in birds. Generally birds do not develop encephalitis except pheasants or emus in which EEEV causes encephalitis with a 50–70% mortality (Luginbuhl et al., 1957). Studies with gallinaceous birds showed that histological lesions are primarily viscerotrophic, rather than neurologic as in mammals (Scott and Weaver, 1989). Young chickens show signs of extensive myocarditis in early experimental infection and heart failure rather than encephalitis is the cause of death (Tyzzer and Sellards, 1941). Beside the heart, other organs such as pancreas and kidney show multifocal necrosis. Additionally, lymphoid depletion has been observed in the thymus and spleen (Griffin, 2001).

Intracerebral infection with EEEV results in fatal disease in monkeys while intradermal, intramuscular, or intravenous inoculations cause disease but does not always result in symptoms of the nervous system. Therefore, the initial viremia and the secondary nervous system infection do not overlap in monkeys when they are infected with peripheral route of administration of EEEV (Wyckoff and Tesar, 1939). Intranasal and intralingual inoculations of EEEV and WEEV also cause nervous system symptoms in monkeys, but are less drastic than intracerebral injections (Wyckoff and Tesar, 1939). After nervous system symptoms were observed, death was certain in young monkeys but occasionally some animals have symptomless infection resulting in a high concentration of circulating antibodies (Wyckoff and Tesar, 1939).

Newborn mice are susceptible to EEEV, which causes neuronal damage. The disease progresses rapidly and results in death (Murphy and Whitfield, 1970). EEEV produces fatal encephalitis in older mice inoculated intracerebrally, while subcutaneous inoculation causes a pantropic infection eventually resulting in encephalitis (Morgan, 1941; Liu et al., 1970).

Guinea pigs and hamsters have also been used as animal models for EEE studies. Subcutaneously inoculating hamsters with EEEV produces lethal with severe lesions of nerve

cells. In addition, parenchyma necroses were observed in the liver and lymphoid organs (Dremov et al., 1978).

Due to its high mortality rate, it is important that an effective vaccine is available for human use. Currently, a formalin-inactivated vaccine prepared from the PE-6 strain of EEEV has IND status and is given only to at-risk laboratory workers. A review of the literature finds very little on new candidate vaccines against EEEV. In fact, one EEEV vaccine construct was described in 1974. The vaccine, designated E_m was prepared by treating EEEV with nitrous acid to a survival of 0.01%. This mutant was described to produce smaller plaques on chick embryo monolayers, but unlike virulent EEEV, did not plaque at all on mouse embryo monolayers. E_m was also determined to be less virulent for mice inoculated both subcutaneously and intraperitoneally. This attenuated vaccine preparation was able to protect mice challenged intraperitoneally but not intracerebrally. Although neutralizing antibodies could not be detected in the E_m-vaccinated mice, antibodies must have played some role in protection after challenge as passive immunization was also able to elicit some protection after challenge (Brown and Officer, 1975).

4.3. Western equine encephalitis virus

Epizootics of viral encephalitis in horses were described in Argentina shortly after the turn of the 20th century, and in 1912 an estimated 25,000 horses died in the central plains of the US (Sabattini et al., 1985). Again, in 1930, a similar outbreak occurred in the San Joaquin Valley of California, causing an estimated 6000 cases of equine encephalitis. It was during this later outbreak that WEEV was isolated from the brains of horses. WEEV was also suspected to cause human encephalitis and in 1938, the virus was recovered from the brain of a child with fatal encephalitis (Griffin, 2001). WEE manifests itself in horses with fever, incoordination, drowsiness and anorexia, leading to prostration, coma, and death in about 40% of infected animals (Doby et al., 1966). As with EEEV, emus also develop symptomatic, often fatal disease characterized by ataxia, paralysis and tremors (Hardy et al., 1997). WEEV also infects other species of birds and often causes fatal disease in sparrows. Mammals are less frequently involved, but bats, jackrabbits, and squirrels can become infected (Ubico and McLean, 1995).

Three other New World viruses, Highlands J, Fort Morgan and Sura viruses, are closely related to WEEV (Calisher et al., 1988). These viruses vary only in their ecological niche and the degree of virulence. Of these related viruses, only WEEV is recognized to cause human disease (Calisher, 1994); however, Highlands J virus has been detected in the brains of dead sparrows and is emerging as an important pathogen in domestic poultry (Whitehouse et al., 2001). WEE occurs throughout western North America as well as sporadically in South America. In the US, WEEV circulates between its mosquito vector and wild birds (Schoeep et al., 2002). Serosurveys and virus isolation have provided evidence of natural infection in chickens and other domestic birds, pheasant, rodents, rabbits, ungulates, tortoises, and snakes (Calisher, 1994; Hardy, 1987). In some areas of South America, most mosquitoes from which WEEV has been isolated feed primarily on mammals, whereas in other areas, antibodies are found primarily in birds (Shope et al., 1966; Weaver et al., 1999). Mechanisms by which the virus can overwinter in endemic areas is unclear. Interseasonal persistence can occur in salt-water marshes, where vertical transmission of WEEV in other mosquito species has been demonstrated (Reisen et al., 1990, 1995). WEEV has caused epidemics of encephalitis in humans, horses, and emus, but the fatality rate of 10% for humans, 20–40% for horses, and 10% for emus is lower than that for EEEV (Ayers et al., 1994). Clinical symptoms are most common in the very young and those older than 50 years (Longshore et al., 1956). Severe disease, seizures, fatal encephalitis, and significant sequelae are more likely to occur in infants and young children (Kokernot et al., 1953; Finley et al., 1955; Earnst et al., 1971). In areas of endemic disease, seroprevalence in humans is fairly common. Phylogenetic studies have determined that WEEV is a naturally occurring recombinant of an EEEV-like and Sinbis virus-like ancestor (Hahn et al., 1988; Levinson et al., 1990; Weaver et al., 1993, 1997).

Ponies, mice, hamsters, and guinea pigs have been used for WEEV studies. Studies with ponies resulted in viremia in 100% of the animals 1–5 days postinoculation. Fever was observed in 7 of 11 animals; only six of these ponies showed signs of encephalitis (Sponseller et al., 1966).

Suckling and adult mice differ with respect to target organ pathology of WEEV infection. After subcutaneous infection with WEEV, suckling mice become sick in 24 h and become moribund or dead in 48 h (Aguilar, 1970). The heart was the only organ in which pathologic changes were observed. Other organs such as lungs, liver, kidney, and brain were entirely disease free. On the other hand, adult mice start to exhibit signs of meningoencephalitis 10 days postinfection and both brain and mesodermal tissues such as heart, lungs, liver, and kidneys were involved (Aguilar, 1970). Furthermore, depending on the dose of virus given, intracerebral and intranasal inoculations of WEEV result in fatal encephalitis in mice, while intradermal and subcutaneous inoculations caused only 50% encephalitic death in mice regardless of the amount of virus given (Liu et al., 1970).

When mice were infected subcutaneously with WEEV, lethargy and ruffled fur, observed on days 4–5 postinfection, were the earliest signs of the illness. Mice were severely ill by day 8 and appeared hunched and dehydrated. Death occurred between days 7 and 14; however, some mice exhibited signs of illness, but recovered between days 10 and 17 postinfection (Monath et al., 1978).

Studies demonstrate that WEEV infection results in fatality in hamsters by all routes of inoculation although the length of the incubation period and the disease duration varied. Progressive lack of coordination, shivering, rapid and noisy breathing, corneal opacity, and conjunctival discharge

resulting in closing of the eyelids were the signs of disease in all cases (Zlotnik et al., 1972). Brain hemorrhages and infarctions were the first changes to occur in the brain and were observed 24 h after intracerebral inoculations. Astrocytic hypertrophy, swelling in the brains of hamsters were observed on day 2 of intraperitoneal and intradermal inoculation and progressed to neuronal necrosis and widespread hemorrhages by days 5–6 (Zlotnik et al., 1972). WEEV also infects guinea pigs (Bianchi et al., 1997). Studies indicate that guinea pigs intraperitoneally inoculated with WEEV results in their fatality, regardless of virus inoculum. The animals begin to exhibit signs of illness on days 3–4 and death occurs between days 5 and 9 (Nalca and Fellows, unpublished data).

As with EEEV and VEEV, the only vaccine available for WEEV has IND status and is given only to at-risk laboratory workers. The vaccine is an inactivated vaccine prepared from a virulent strain of the virus. This vaccine is not strongly immunogenic and takes several boosters to induce even low levels of antibody. In fact, some vaccinees never seroconvert. Thus, the development of a new candidate vaccine for WEEV would be very beneficial. A review of the literature describes very limited studies investigating the development of new WEEV vaccines. In fact, only one study has been published in recent years and that construct was prepared as a potential useful vaccine for both WEEV and EEEV. Chimeric vaccines often result in attenuated viruses and thus may provide a useful alternative approach for creating genetically engineered vaccine candidates (Kuhn et al., 1996). In fact, as noted above, chimeric vaccines have proven successful in protecting laboratory animals from challenge with dengue, yellow fever, and JE viruses (Monath et al., 1992; Bray et al., 1996; Chambers et al., 1999). Because WEEV and EEEV are very similar, a study was undertaken to make chimeric virus clones from two full-length cDNA clones of WEEV and the structural gene region of EEEV. These chimeric clones were designated pMWE1000 and pMWE2000, and they contain the nonstructural protein domain of WEEV and the structural protein domain of EEEV. These clones were used in vaccination studies with mice. Both chimeric viruses were significantly attenuated when compared to the parental virus strains, causing only sporadic deaths in groups of mice receiving high doses of the vaccine ($>10^5$ pfu). However, at a lower concentration of 10^3 pfu, no vaccination-associated deaths were observed. This is in contrast to the more virulent parental isolates of EEEV and WEEV, which killed 70 and 100%, respectively, of infected mice. Subsequently, when the vaccinated mice were challenged with virulent EEEV strain FL91-4679 or virulent WEEV strain Cba 87, the chimeric vaccines provided substantial protection. The protection ranged from 50 to 90% and was dependent upon the dose of the vaccine. Those mice challenged with the virulent EEEV responded with solid levels of neutralizing antibodies. In contrast, the mice surviving challenge with virulent WEEV produced fewer neutralizing antibodies. The residual virulence in these chimeric constructs was initially a concern to investigators. However, as the attenuated phenotype of the chimeric viruses was the result of numerous nucleotide and amino acid changes that altered virus–cell interaction, it was felt that the chimeras would have little chance of reversion to virulence, yet would be able to provide many advantages of a live virus vaccine (Schoeep et al., 2002). Perhaps with further investigations into other animal models and newer methods of vaccine delivery, the success of these potential vaccine candidates will be even greater.

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